

10/561902

IAP9 Rec'd PCT/PTO 21 DEC 2005

Modulation of Osteoblast Activity by Fhl2

The present invention provides means and methods for regulating the activity of osteoblasts. The invention further relates to methods for identifying compounds that are useful in the treatment of osteoporosis.

Bone is an organ of special importance offering resistance to mechanical stress and protecting internal organs from trauma. Bone is built by only three different cell types. During embryonic development chondrocytes produce a blueprint of cartilage for most bones. Successively, the cartilage is replaced by bone substance. This is an extracellular matrix mostly produced by osteoblasts which calcifies under its control. Therefore osteoblasts represent the bone forming cells. The formation of bone is yet not static. During the entire life osteoclasts degrade bone substance which subsequently will be replaced by the activity of osteoblasts thereby absorbing the varying burden of the skeleton (Karsenty and Wagner, 2002). An equilibrium of the activity of osteoblasts and osteoclasts exists in a full-grown human before the entrance into old age. However, if the number and/or activity of osteoblasts decreases or if the number and/or activity of osteoclasts increases expanded bone loss will be the outcome (osteoporosis). The reduced bone substance causes an increased risk for fractures (Teitelbaum, 2000).

BEST AVAILABLE COPY

Osteoporosis can have quite different reasons. The most important one is the increase of the osteoclast cell population and its activity in postmenopausal women due to decreased estrogen synthesis. Besides that, differentiation, activity and apoptosis of osteoclasts as well as the presence of some extracellular matrix proteins play an important role. The knowledge about loss of bone substance caused by decreased osteoblast activity is still surprisingly small. This is very well underlined by the lack of supporting animal models.

Osteoporosis is of outstanding individual and economic significance. It is the most prominent degenerative disease in the western world. The economic damage caused by fractures due to osteoporosis only in the USA is assessed to be about 10 to 15 billion \$ (NIH Consensus Development Panel on Osteoporosis Prevention, 2001).

Approaches to fight osteoporosis on the pharmacological level lie in the increase of serum calcium by supplementation of nutrition. Also specific manipulation of the estrogen signaling cascade by administration of estrogen or the modulation of the estrogen receptor (e.g. Raloxifene) is widely used in females. Inhibition of bone turnover by bisphosphonates (e.g. Alendronate) or the inhibition of bone resorption by calcitonin can be an alternative strategy.

Most of the known therapies try to inhibit osteoclast activity, e.g., stop bone resorption and continuing damage. Hence, there is an ongoing need for a therapy that could lead to the reconstruction of bone substance resembling normal bone mineral density.

The inventors surprisingly found that Fhl2 significantly increases the transcriptional activity of the gene Runx2. In addition, the interaction of Fhl2 protein and Runx2 protein regulates the activity of target genes, e.g., osteocalcin, and therefore the activity of osteoblasts.

As used herein, the term "activity of osteoblasts" denotes the capability of osteoblasts to form extracellular matrix. This activity can be determined *in vitro* by the following assay: Synthesis by the cells of extracellular matrix is induced by adding 5 mM β -glycerophosphate and 100 μ g/ml medium ascorbic acid. After incubation, the amount of calcified matrix is determined by von Kossa staining and quantification of the stained area (Bancroft, J.D. and A. Stevens. 1996. Theory and practice of histological techniques. Churchill Livingstone, New York, NY).

Osteoblast activity can be determined *in vivo* as described in Example 1.

"Four and a Half LIM Domains 2" (Fhl2) belongs to a subclass of the "LIM-only" proteins of the super-family of LIM-domain proteins. Family members are characterized by the presence of a single or several LIM domains. LIM proteins play an important role in determination, differentiation and proliferation of cells. Fhl2 as well as Fhl1, -3, -4 and "Activator of Crem in Testis" (Act) are characterized by four complete and a single half LIM domain. The LIM domain is a cysteine-rich motif that can bind two zinc ions and mediates protein-protein interactions.

Besides expression in the heart muscle FHL2 could be detected in the epithelial cells of the prostate where it very well co-localizes with the activated androgen receptor (AR) in the nucleus (Müller et al. 2002). The AR belongs to a family of steroid receptors which are capable of directly binding to so called response elements in the promoter of target genes. Their transcriptional activity can be influenced by cofactors. Fhl2 can directly bind to AR thereby functioning as a tissue specific coactivator (Müller et al. 2000). The translocation of Fhl2 from the cytoplasm into the nucleus and the super-activation of the transcriptional activity of the AR is specifically triggered by Rho-GTPases. This way, extracellular signals can be translated into altered gene expression via the Rho-signaling cascade and Fhl2. The aberrant regulation of the AR activity plays a decisive role in the formation and progression of tumors in the prostate. Indeed, over-expression of Rho-GTPases in prostate carcinomas and the correlation of nuclear Fhl2 localization with the malignancy of the tumor has been shown. (Müller et al., 2002).

The present invention therefore relates to a method for identifying a compound that promotes the activity of osteoblasts, comprising:

- (a) contacting a test compound with at least one cell *in vitro*;
- (b) determining an activity of the Fhl2 gene or Fhl2 protein;
- (c) comparing the activity determined in (b) to the activity of the Fhl2 gene or Fhl2 protein in at least one control cell that has not been contacted with the test compound; and
- (d) selecting the test compound if the activity measured in (b) is significantly different from that in the at least one control cell.

The method may further comprise one or more of the following steps:

- determining whether the test compound is capable of promoting the activity of osteoblasts; and optionally selecting the test compound if it is capable of promoting the activity of osteoblasts;
- determining whether the test compound is capable of modulating the bone formation rate in a non-human test animal; and optionally selecting the test compound if it is capable of modulating the bone formation rate in a non-human test animal;
- determining whether the test compound is capable of promoting the formation of extracellular bone matrix in a non-human test animal; and optionally selecting the test compound if it is capable of promoting the formation of extracellular bone matrix in a non-human test animal;
- determining whether the test compound is capable of increasing the expression of the Runx2 gene; and optionally selecting the test compound if it is capable of increasing the expression of the Runx2 gene;
- using the test compound for the manufacture of a medicament for the treatment of bone disease, e.g., osteoporosis.

The term "activity of the Fhl2 gene or Fhl2 protein" includes but is not limited to, the expression of the Fhl2 gene (e.g., transcription and/or translation); the ability of the Fhl2 protein to translocate into the cell nucleus; the capability of the Fhl2 protein to interact with the Runx2 protein; the capability of the Fhl2 protein to stimulate the expression of other genes; and the like.

In one embodiment the test compound is selected if the activity of the Fhl2 gene or Fhl2 protein is significantly higher than that of the control. In another embodiment, the test compound is selected if the activity of the Fhl2 gene or Fhl2 protein is significantly lower than that of the control.

Suitable cell types that can be used include, but are not limited to, primary osteoblasts; MC3T3-E1 cells, ROS17 cells; and U2-OS cells. Preferably, the cells are osteoblasts, e.g., primary osteoblasts. Osteoblasts can be obtained as primary cells from the calvaria of newborn mice and subsequently cultured. By adding 5 mM glycerophosphate and 100 µg/ml medium ascorbic acid the production of calcifying extracellular matrix can be induced (Zhang et al. 1997; Journal of Biological Chemistry 272, 110-116). The cell line MC3T3-E1 is an established mouse osteoblast cell line.

In a specific embodiment, cells, preferably animal cells, and more preferably mammalian cells that have been manipulated to be missing all or essentially all of an activity of the Fhl2 protein can be used. Such cells may be derived from a transgenic non-human animal described *infra*. The transgenic non-human animal is characterized by a decreased level of expression of the Fhl2 gene relative to that of the corresponding wild-type animal. The transgenic non-human animal may be an Fhl2-deficient knockout mouse.

Usually, more than one cell is used. In that case, a population of cells in cell culture may be grown under suitable conditions. The test compound can be added to the population of cells, e.g., by adding it to the cell culture medium. Methods of culturing cells are known to those skilled in the art. The control cells are usually of the same cell type as the cells to which the test compound has been added. They are usually cultured under substantially identical conditions except for addition of the test compound.

In a first embodiment the method comprises:

- (a) contacting a test compound with at least one cell *in vitro*;
- (b) measuring the level of *Fhl2* expression in the cell(s);
- (c) comparing the level of *Fhl2* expression measured in (b) to the level of *Fhl2* expression in at least one control cell that has not been contacted with the test compound; and
- (d) selecting the compound if the level of *Fhl2* expression measured in (b) is higher than that in the at least one control cell.

The step of determining the level of expression of the Fhl2 gene includes, but is not limited to, measuring the amount and/or concentration of Fhl2 mRNA and/or measuring the amount and/or concentration of Fhl2 protein in cells or tissue.

The amount of Fhl2 mRNA in cells or tissue can be measured by methods known to those skilled in the art, e.g., by RT-PCR (Du et al., 2002, *Biochimica et Biophysica Acta* 1577, 93-101) or a Northern or Western Blot protocol (Müller et al. 2000; *EMBO J.* 19:359-369). The nucleotide sequence of human Fhl2 as shown in SEQ ID NO:1 can be used to design suitable primers and/or probes capable of hybridizing to Fhl2 mRNA or Fhl2 cDNA under stringent conditions as described herein. It is contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long

contiguous sequence of SEQ ID NO:1 will find particular utility. Longer contiguous identical or complementary sequences, e.g., those of at least 20, 30, 40, 50, 100, 200, 500 and even up to full length sequences will also be of use in certain embodiments. This embodiment is particularly useful if human cells or human tissue is used. If cells or tissue from non-human origin are employed the corresponding non-human nucleotide sequence of the Fhl2 gene can be used for designing suitable primers or probes. For example, when mouse cells are used, it is preferred that the primers or probes are derived from the mouse Fhl2 sequence (Accession No. NM_010212).

The stringent conditions referred to herein include, for example, hybridization at 20 mM to 40 mM NaCl at a temperature of about 50°C to about 70°C, preferably at about 60°C to 65°C.

The amount of Fhl2 protein in cells or tissue can be measured by methods generally known in the art, e.g., by immunochemical methods using antibodies recognizing the Fhl2 protein. Such methods include, but are not limited to, Western blots, dot blots, ELISA protocols, histochemical methods, and the like. Antibodies recognizing Fhl2 protein can be prepared and optionally purified using methods known to those skilled in the art. Methods for preparing and isolating polyclonal and monoclonal antibodies are well known in the art. See, for example, Current Protocols in Immunology, Cooligan et al. (eds.), National Institutes of Health, John Wiley and Sons, Inc., 1995; Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, NY, USA, 1989; and Hurrell, J.G.R., Ed., Monoclonal Hybridoma Antibodies: Techniques and Applications, CRC Press, Inc., Boca Raton, Florida, USA, 1982. The amino acid sequence of human Fhl2 protein as shown in SEQ ID NO:2 can be used in order to design suitable antigens for the generation of antibodies. If cells from non-human origin are employed suitable antigens can be derived from the amino acid sequence of the corresponding non-human Fhl2 protein. Monoclonal and polyclonal antibodies against Fhl2 protein have been described in Müller et al. (2000) EMBO J. 19, 359-369 and in Müller et al. (2002) EMBO J. 21, 736-748.

Independently of step (a) and (b), the level of Fhl2 expression in control cells can be determined. The control cells have not been contacted with the test compound. The control cells have preferably been cultured under conditions which are identical to the conditions under which the cells that have been contacted with the test compound have been cultured, except for addition of the test compound. Usually, the compound is selected if the level of Fhl2 expression measured in (b) is significantly higher than that in the control cells.

Preferably, the compound is selected if the level of Fhl2 expression measured in (b) is at least 10% or at least 20% or at least 50% or at least 100% higher than that in the control cells.

In a second embodiment, the method for identifying a compound that promotes the activity of osteoblasts comprises:

- (a) contacting at least one cell with a test compound *in vitro*;
- (b) measuring the amount of Fhl2 protein in the nucleus of the cell(s);
- (c) comparing the amount of Fhl2 protein measured in (b) to the amount of Fhl2 protein in the nucleus of at least one control cell that has not been contacted with the test compound; and
- (d) selecting the compound if the level of Fhl2 protein measured in (b) is higher than that (in the control cell(s)).

The amount of Fhl2 protein in the nucleus of cells may be determined by one of the following methods: The intracellular localization of Fhl2 can be detected by immune histochemistry using an anti-Fhl2 antibody as described in Müller et al., 2002, EMBO J. 21:736-748. In another embodiment, the amount of Fhl2 in a nuclear extract and in a total cell extract can be determined (see, e.g., Müller et al., 2002, EMBO J. 21:736-748).

In a third embodiment, the method for identifying a compound that promotes the activity of osteoblasts comprises:

- (a) contacting at least one cell with a test compound *in vitro*;
- (b) determining the level of interaction between Fhl2 protein and Runx2 protein in the at least one cell;
- (c) comparing the level of interaction determined in (b) to the level of interaction between Fhl2 protein and Runx2 protein in at least one control cell that has not been contacted with the test compound; and
- (d) selecting the compound if the level of interaction measured in (b) is significantly different from that in the at least one control cell.

Step (b) includes methods known to those skilled in the art, e.g., co-immunoprecipitation and pulldown assays as described herein (see Example 2). Direct or indirect interaction of Fhl2

and Runx2 can be determined in a transfection assay wherein a reporter gene is employed. For example, a reporter gene operably linked to a promoter which binds to Runx2 may be transfected together with Runx2 DNA and Fhl2 DNA (See Examples).

In a specific embodiment, the compound is selected if the level of interaction determined in (b) is significantly higher than that in the at least one control cell. Preferably, the level of interaction determined in (b) should be at least 10% or at least 20% or at least 50% or at least 100% higher than that in the at least one control cell.

In another specific embodiment, the compound is selected if the level of interaction measured in (b) is significantly lower than that in the at least one control cell. Preferably, the level of interaction determined in (b) should be less than 90% or 80% or 50% or 25% of that in the at least one control cell.

The invention concerns a compound that is useful in the treatment of a bone disease wherein the compound is capable of promoting osteoblast activity by enhancing the expression of the Fhl2 gene, promoting the translocation of Fhl2 protein in the nucleus and/or modulating the interaction between Fhl2 protein and Runx2 protein. The compound is obtainable by a method described herein. In one embodiment, the compound is capable of enhancing signals mediated by Rho proteins. Examples of compounds that influence the Rho signalling pathway include, but are not limited to, lysophosphatidic acid (LPA) and sphingosine-1-phosphate (SPP).

The invention further concerns the use of a compound according to the invention for the manufacture of a medicament for the treatment of a bone disease, e.g., osteoporosis.

Another aspect of the present invention is a method of diagnosing a bone disease, comprising:

- (a) determining *in vitro* the level of expression of the *Fhl2* gene in tissue from an individual; and
 - (b) comparing the level determined in (a) to the level of expression of the *Fhl2* gene in control tissue;
- so that if the level determined in (a) is lower than that of the control, the individual is diagnosed as exhibiting the bone disease.

The level of Fhl2 expression may be determined as described supra, e.g., by measuring the amount/concentration of mRNA in the cells or by measuring the amount/concentration of Fhl2 protein present in the cells.

The determination can be carried out using a tissue sample derived from an individual. Preferably, the tissue sample comprises bone material, more preferably the tissue sample comprises osteoblasts. The tissue sample may be obtained through biopsy from an individual. In a specific embodiment, osteoblasts are enriched after having obtained the biopsy and prior to determining the level of expression of the Fhl2 gene.

Independently of step (a), one can determine *in vitro* the level of expression of the Fhl2 gene in tissue or cells from a healthy control individual. Preferably, the type of tissue or cells to be examined as control is the same type of tissue or cells as that used in step (a). It is important that the control individual does not suffer from a bone disease, e.g., osteoporosis.

From the amount of Fhl2 mRNA or protein one can derive the concentration of Fhl2 mRNA or protein, given as g/(number of cells), mol/(number of cells), g/(g cell mass), mol/(g cell mass), or the like.

Usually, the individual is diagnosed as exhibiting the bone disease, if the level determined in step (a) is lower than that of the control. In specific embodiments, the individual is diagnosed as exhibiting the bone disease, if the level of Fhl2 expression (e.g., the amount or concentration of the Fhl2 mRNA or the amount or concentration of the Fhl2 protein) determined in (a) is less than 90% or 80% or 70% or 60% or 50% of the level of Fhl2 expression of the control.

Preferably, the bone disease to be diagnosed is characterized by a decreased bone mass relative to that of non-diseased bone. Most preferably, the bone disease to be diagnosed is osteoporosis.

The present invention further relates to the use of an Fhl2 nucleic acid for the manufacture of a medicament for the treatment of bone disease. As used herein, the phrase "Fhl2 nucleic acid" denotes one of the following nucleic acids:

- (i) Polynucleotides comprising a sequence which has an identity of at least 50% to the sequence as shown in SEQ ID NO:1. Preferably, the identity is at least 60%, more preferably at least 70%, still more preferably at least 80%, still more preferably at least 90%. Most preferably, the polynucleotide comprises a sequence as shown in SEQ ID NO:1.
- (ii) Polynucleotides hybridizing to the sequence as shown in SEQ ID NO:1 under stringent conditions.
- (iii) Polynucleotides comprising a sequence which encodes a polypeptide having an amino acid sequence which has an identity of at least 50% to the amino acid sequence as shown in SEQ ID NO:2. Preferably, the identity is at least 60%, more preferably at least 70%, still more preferably at least 80%, still more preferably at least 90%. Most preferably, the polynucleotide comprises a sequence which encodes a polypeptide having an amino acid sequence as shown in SEQ ID NO:2.

In one embodiment the bone disease is characterized by a decreased bone mass relative to that of non-diseased bone. The bone disease may be selected from the group consisting of osteoporosis, osteopenia, and Paget's disease. Preferably, the bone disease is osteoporosis.

The Fhl2 nucleic acid may be used in a gene therapy protocol to increase the activity of osteoblasts. If an individual has a mutated or absent Fhl2 gene or shows a decreased level of Fhl2 expression, the Fhl2 nucleic acid can be introduced into the cells of the individual. In one embodiment, a gene encoding a Fhl2 polypeptide is introduced in vivo in a viral vector. Such vectors include an attenuated or defective DNA virus, such as, but not limited to, herpes simplex virus, papilloma virus, Epstein-Barr virus, adenovirus, adeno-associated virus, and the like. The effective viruses which entirely or almost entirely lack viral genes are preferred. A defective virus is not effective after the introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Examples of particular vectors include, but are not limited to, a defective herpes simplex virus 1 vector (Kapli et al., *Molec. Cell. Neurosci.* 2:320-30, 1991); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet et al., *J. Clin. Invest.* 90:626-30, 1992; and a defective adeno-associated virus vector (Samulski et al., *J. Virol.* 61:3096-101, 1987; Samulski et al., *J. Virol.* 63:3822-8, 1989).

In another embodiment, a Fhl2 nucleic acid can be introduced in a retroviral vector, e.g., as described in Anderson et al., U.S. patent No. 5,399,346; Mann et al. Cell 33:153, 1983; Temin et al., U.S. patent No. 4,650,764; Temin et al., U.S. patent No. 4,980,289; Markowitz et al., J. Virol. 62:1120, 1988; Temin et al., U.S. patent No. 5,124,263; WO 95/07358; and Kuo et al., Blood 82:845, 1993. Alternatively, the vector can be introduced by lipofection in vivo using liposomes. Synthetic cationic lipids can be used to prepare liposomes for in vivo transfection of a gene encoding a marker (Felgner et al., Proc. Natl. Acad. Sci. USA 84:7413-7, 1987; Mackey et al., Proc. Natl. Acad. Sci. USA 85:8027-31, 1988). The use of lipofection to introduce exogenous genes into specific organs in vivo has certain practical advantages. Molecular targeting of liposomes to specific cells represents one area of benefit. More particularly, directing transfection to particular cells represents one area of benefit. For instance, directing transfection to osteoblasts would be particularly advantageous. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (e.g., hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

It is possible to remove the target cells from the body; to introduce the vector as a naked DNA plasmid; and then to re-implant the transformed cells into the body. Naked DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, e.g., transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun or use of a DNA vector transporter. See, e.g. Wu et al., J. Biol. Chem. 267:963-7, 1992; Wu et al., J. Biol. Chem. 263:14621-4, 1988.

In another embodiment, the invention pertains to a method for identifying Fhl2-regulated target genes. The method may comprise the step of comparing a cDNA pool from a first population of cells with a cDNA pool from a second population of cells wherein in the first population of cells Fhl2 transcription or nuclear translocation has been induced. In the second population of cells Fhl2 transcription or nuclear translocation has not been induced. Microarrays may be used for comparing the cDNA pools.

A genetically modified non-human animal characterized by a decreased level of expression of the Fhl2 gene relative to that of the non-modified non-human animal may be used as an osteoporosis animal model. The level of expression of the Fhl2 gene in the modified non-human animal may be less than 90%, preferably less than 75%, more preferably less than

50% and still more preferably less than 25% of the level of Fhl2 expression in the non-modified non-human animal. The amount or concentration of Fhl2 mRNA or Fhl2 protein in bone tissue may be a measure for the level of expression of the Fhl2 gene.

Most preferably, the non-human animal completely lacks expression of functional Fhl2 protein. In that embodiment, the Fhl2 gene may have been deleted or rendered non-functional.

It is also preferred that the non-human animal is a non-human mammal, more preferably the non-human animal is a rodent, most preferably, the non-human animal is a mouse.

The non-human animal may have been manipulated to be missing all or essentially all of an activity of one or more specific gene/allele products. In a preferred embodiment, the non-human animal has been manipulated so as not to express functional Fhl2 protein.

In addition, according to the invention cells, preferably animal cells, and more preferably mammalian cells that have been manipulated to be missing all or essentially all of an activity of the Fhl2 protein, may be useful as osteoporosis model systems. The cells may be human or non-human cells. Preferably, the cells are bone cells, e.g., osteoblasts.

The invention provides methods of making and using these cells and non-human animals. The knockout animals and/or corresponding cells of the present invention can be manipulated to be incapable of expressing a functional protein from one or more specified alleles and/or genes by any means known in the art. For example, a knockout animal and/or corresponding cell can be manipulated to comprise a disruption in an endogenous allele and/or gene, e.g., the Fhl2 gene; thereby preventing the expression of the corresponding functional protein. Alternatively, a knockout animal and/or corresponding cell can be manipulated to comprise a dominant mutant allele. Fhl2 knockout mice have been described in Kong et al. 2001, Circulation 103, 2731-2738.

In yet another embodiment, a knockout animal and/or corresponding cell can be treated with one or more antisense nucleic acids for one or more specific gene(s) thereby inhibiting the expression of the specific gene(s). In still another embodiment, the gene(s) encoding the specific functional protein(s) can be constructed such that the expression of the protein(s) is under the control of an inducible promoter, and this expression is conditionally repressed. In

still another embodiment, the cell and/or non-human animal is treated with/administered inhibitory compound(s) that prevent the expression and/or activity of the Fhl2 protein.

The invention includes the use of a non-human knockout animal that comprises a homozygous disruption in its endogenous Fhl2 gene as an osteoporosis model.

The non-human animal preferably shows decreased bone formation rate relative to that of the corresponding wild-type animal. The bone formation rate may be decreased by at least 5%, preferably at least 10%, most preferably at least 15%, compared to the corresponding wild-type animal. The bone formation rate may be determined as described in Example 1.

The non-human animal preferably shows a decrease of the amount of the calcified extracellular matrix. This decrease may concern the vertebral bodies of the non-human animal. The amount of the calcified extracellular matrix may be decreased by at least 10%, preferably by at least 20%, more preferably by at least 25%, most preferably by at least about 30%, compared to the amount of the calcified extracellular matrix in a corresponding wild-type animal. The decrease can be present in male or in female animals.

According to the invention also a cell from the transgenic non-human animal of the invention may be useful. Preferably the cell is from the knockout mouse and has the same genetic background as the knockout mouse.

"Gene targeting" is a type of homologous recombination that occurs when a fragment of genomic DNA is introduced into a mammalian cell and that fragment locates and recombines with endogenous homologous sequences as exemplified in US patent No. 5,464,764 and US patent No. 5,777,195, the contents of which are incorporated herein by reference.

As used herein, the term "knockout animal" denotes an animal that has been manipulated to be irreversibly missing all or "essentially all" of an activity of one or more specific gene/allele product(s) relative to the corresponding wild type animal. In a particular embodiment of this type, the knockout animal contains within its genome a specific gene/allele that has been inactivated by a method such as gene targeting. As used herein, the term "knockout animal" can therefore include the heterozygote animal (e.g., one defective allele and one wild-type allele), a homozygous animal (e.g., two defective alleles) or an animal having more than one

gene inactivated. In a particular embodiment of the present invention, a knockout animal is a knockout mouse that has both alleles of the *Fhl2* gene inactivated.

The non-human animal described supra can be used as an *in vivo* osteoporosis model. The non-human animals offer the unique possibility to test the effect of pharmacological substances on the activity of osteoblasts and therefore their efficacy in osteoporosis treatment. The invention relates to the use of an *Fhl2*-deficient non-human animal for the development of a medicament for the treatment of bone disease, e.g., osteoporosis.

More specifically, the invention relates to a method for identifying a compound that promotes the activity of osteoblasts, comprising:

- (a) administering a test compound to a transgenic non-human animal characterized by a decreased level of expression of the *Fhl2* gene relative to that of the corresponding wild-type animal;
- (b) determining an activity of the *Fhl2* gene or *Fhl2* protein;
- (c) comparing the activity determined in (b) to the activity of the *Fhl2* gene or *Fhl2* protein in a control animal that has not been contacted with the test compound; and
- (d) selecting the test compound if the activity measured in (b) is significantly different from that in the control animal.

The various embodiments of *in vitro* methods for identifying a compound that promotes the activity of osteoblasts as described supra apply to this *in vivo* method.

The present invention offers a completely new approach to specifically fight osteoporosis by the possibility of the well-defined modulation of osteoblast activity. Contrary to the known therapies (inhibition of osteoclast activity; that is. stop of bone resorption and continuing damage) the present invention offers the possibility to develop methods and substances that could lead to the reconstruction of bone substance resembling normal bone mineral density. So far only one transcription factor (*Runx2*) is known to modulate activity of fully differentiated osteoblasts (Ducy et al., 1999). The inventors showed that *Fhl2* significantly increases the transcriptional activity of *Runx2*. The interaction of *Fhl2* and *Runx2* regulates the activity of target genes (e.g. osteocalcin) and therefore the activity of osteoblasts. This observation is corroborated *in vivo* by the osteoporosis phenotype of *Fhl2*-deficient mice. *Fhl2*^{-/-} mice exhibit a decreased osteoblast activity.

The various embodiments of the invention described herein may be combined.

The pharmacological intervention in the Fhl2-mediated signaling cascade offers a new attempt to regulate osteoblast activity directly. Possible pharmacological targets are:

- 1.) Protein-protein interaction required for the activation of Fhl2 (e.g. modulation of the Runx2-Fhl2 interaction).
- 2.) Substances modulating the translocation of Fhl2 from the cytoplasm into the nucleus (e.g. modulators of the Rho signaling cascade; Müller et al., 2002).
- 3.) Fhl2-regulated target genes in osteoblasts.
- 4.) The *Fhl2*-deficient mice are a new in vivo osteoporosis model. *Fhl2*^{-/-} mice offer the unique possibility to test the effect of pharmacological substances in regards to the activity of osteoblasts and therefore their efficacy in osteoporosis treatment.

Figure 1: Expression of FHL2 in bone of adult mice.

The endogenous expression of FHL2 in bone, bone marrow and periosteum of the tibia is reflected through the LacZ stain in a heterozygote mouse. The LacZ gene is under the control of the endogenous Fhl2 promoter.

Figure 2: Decreased calcification of the extracellular matrix in *Fhl2*^{-/-} bone.

Von Kossa stain of vertebral bodies from an adult wild-type (left) and *Fhl2*^{-/-} (right) mouse. The comparison reflects a decrease of 30% in the calcified extracellular matrix (black) in mutant mice.

Figure 3: Number of osteoblasts and osteoclasts.

Despite osteoporosis, the number of osteoblasts and osteoclasts is not altered in adult vertebral bodies of wild-type and *Fhl2*^{-/-} mice.

Figure 4: Activity of osteoclasts is not increased.

Quantification of osteoclast activity by measurement of the corresponding products of bone decomposition in the urine. The amount of pyridinium crosslinks is determined in relation to the total protein content. Osteoclast activity is not altered in *Fhl2*-deficient mice compared to wild-type animals.

Figure 5: Decreased bone formation rate in Fhl2^{-/-} mice.

Dynamic histomorphometric analysis after *in vivo* labeling with the fluorescent dye calcein in an interval of 7 days. The distance (yellow bar) of the mineralization fronts (green) in representative sections of the tibia of adult wild-type and Fhl2^{-/-} mice reflect osteoblast activity. The osteoblast activity is decreased by 19% in mutant mice.

Figure 6: Fhl2 is a coactivator for Runx2 using an artificial promoter.

Concentration-dependent increase of the transcriptional activity of Runx2 by Fhl2 in transient transfections in Cos-7 cells.

Figure 7: Fhl2 enhances the effect of Runx2 on the human osteocalcin promoter.

Concentration-dependent increase of the transcriptional activity of Runx2 by Fhl2 in transient transfections in Cos-7 cells. The human Osteocalcin promoter contains two binding sites for Runx2.

Figure 8: Physical interaction of Fhl2 and Runx2.

Bacterially expressed fusion protein of Fhl2 and GST and *in vitro* translated and labeled Runx2 can be detected in a pulldown. Runx2 does not unspecifically interact with GST alone.

Figure 9 *In vivo* interaction of Fhl2 with Runx2

Co-Immunoprecipitation of FLAG-tagged Fhl2 and Runx2.

Figure 10 shows the nucleotide sequence of the human Fhl2 gene (Accession No. NM_001450; SEQ ID NO:3). The sequence includes the coding sequence as well as 5'- and 3'-noncoding sequences.

Figure 11 shows the coding sequence of the human Fhl2 gene (A; SEQ ID NO:1) and the amino acid sequence encoded by the human Fhl2 gene (B; SEQ ID NO:2).

The following examples further illustrate the invention.

Example 1: Analysis of Fhl^{-/-} mice

Fhl2-deficient mice can be generated as described in Kong et al. 2001, Circulation 103: 2731-2738.

Histology and histomorphometry:

For LacZ stain bone was fixed in 0.2% formaldehyde in 0.1M PIPES buffer pH 6.9, 2mM MgCl₂, 5mM EGTA overnight, incubated in 30% sucrose containing 2mM MgCl₂ and embedded in OCT on dry ice. Cryosections were stained overnight with X-Gal, washed and mounted.

Histological analysis was performed on undecalcified sections stained with von Kossa reagent and counterstained with Kernechtrot (Amling et al., 1999). In vivo double labeling has been performed as described (Amling et al., 1999). Two calcein injections (25mg/kg body weight) were applied in an interval of seven days. The mice were sacrificed two days later. The bones were dissected and fixed in 4% PBS-buffered formaldehyde. After embedding in methylmethacrylate and sectioning at 6µm, a blind analysis of the unstained samples was conducted under fluorescent light. Histomorphometric analyses were performed according to standard protocols (Parfitt et al., 1987) using the Osteomeasure Analysis System (Osteometrics, Atlanta).

Results:

Analysis of the Fhl2 distribution in the mouse revealed that Fhl2 is also expressed in the adult bone (Figure 1). No alteration of the structure or size of the skeleton could be detected, which would have been an indication of aberrant pattern formation and/or modified growth.

The comparison of vertebral bodies, however, revealed a dramatic decrease of the amount of the calcified extracellular matrix by 30% in male and female Fhl2-deficient animals (Figure 2). The number of osteoblasts as well as the number of osteoclasts in 'knockout'-mice is not altered in comparison to wild-type mice (Figure 3). Analysis of the osteoclast activity also exhibited no significant abnormality (Figure 4). An *in vivo* double labeling using a fluorescent dye in an interval of seven days revealed an articulate decreased amount of newly build

extracellular matrix (Figure 5). This difference in the bone formation rate derived from the significantly diminished activity of Fhl2-deficient osteoblasts accounted for 19%.

Example 2: Cell culture experiments

Materials and Methods

Plasmids and reagents

The following plasmids were described previously: pCMX Fhl2, pGEX Fhl2 (Müller *et al.*, 2000); pCMV5 Cbfa1 (Meyers *et al.*, 1995); p6OSE2-luc (Ducy and Karsenty, 1995); hOC-luc (Yeung *et al.*, 2002). The pGEM Cbfa1 was a kind gift of Florian Otto (Freiburg Medical Center). Osteoclast activity was determined using the Metra PYD EIA and Metra Creatinine Assay kits (Quidel).

Cell lines and transfections

Cos-7 cells were cultured in DMEM supplemented with 10% fetal calf serum. Transient transfection assays were carried out in 12-well plates (1×10^5 cells per well). Cells were transfected using the calcium phosphate precipitation method as described (Müller *et al.*, 2000). Total amount of transfected DNA was kept constant (4 µg) by adding the corresponding amounts of empty expression plasmids and pUC18. 500 ng of reporter plasmids were transfected per well. Luciferase activity was assayed 24-30 h after transfection as described (Müller *et al.*, 2000).

GST pulldown assays

Expression of the GST fusion protein (Pharmacia) and the in vitro transcription-translation reactions (Promega) were performed according to the manufacturer's instructions. GST pulldown assays were performed as described (Dörflinger *et al.*, 1999) using buffer containing 150 mM KCl at 37°C. 10% of the in vitro translated protein was loaded as input.

Coimmunoprecipitation assays and Western blot analyses

293 cells were transfected with 5µg of pCMV5 Cbfa1 and 5µg pCMX-Flag-Flirt1. Following preclearing with a 40µl 1:1 slurry of GammaBind-Sepharose (Pharmacia), supernatants were incubated for 2.5h with M5 α-Flag antibody (Sigma) and protein extract of the transfected cells. Beads were washed 5 times with WB (10mM Tris-HCl, pH 8.0, 250mM NaCl, 0.5% NP-40, 0.1µg/µl bovine serum albumin, 0.5mM Pefabloc) and analyzed on a 10% SDS gel. Western blots were decorated with an α-PEBP2αA1 (Santa Cruz) antibody. Secondary antibody and chemoluminescence procedure was performed according to the manufacturer (Amersham).

Results

Runt related protein 2 (Runx2, also referred to as Cbfa1, Osf2, AML3 or PEBP2αA1) is essential for the differentiation of precursors to become mature osteoblasts. Hence, *Runx2*-deficient mice lack bone completely whereas haplo-insufficiency caused cleidocranial dysplasia in men and mice. Moreover, it could be demonstrated in transgenic mice that Runx2 increases osteoblast activity without affecting osteoclast number or activity (Ducy et al. 1999).

In transient transfections in cell culture Fhl2 can increase the transcriptional activity of Runx2 by the factor of three. This super-activation of Runx2 by Fhl2 could not only be demonstrated using an artificial promoter comprising six copies of binding sites for Runx2 in front of a luciferase reporter gene (Figure 6) but also using the promoter of the human osteocalcin gene, a natural target for Runx2 (Figure 7).

The superactivation of Runx2 by Fhl2 could be accomplished either by direct interaction of both proteins or indirectly. In a pulldown it could be shown that Fhl2 physically interacts with Runx2 *in vitro* (Figure 8).

The interaction of Fhl2 and Runx2 can also be shown *in vivo* in immunoprecipitations by transfection of 293 cells with FLAG-tagged Fhl2 and Runx2.

Literature

- Amling M., Priemel M., Holzmann T., Chapin K., Rueger J.M., Baron R., and Demay M.B. (1999). Rescue of the skeletal phenotype of vitamin D receptor-ablated mice in the setting of normal mineral ion homeostasis: histomorphometric and biomechanical analyses. *Endocrinology*. 140:4982-4987.
- Dörflinger U., Pscherer A., Moser M., Rummele P., Schüle R., and Buettner R. (1999). Activation of somatostatin receptor II expression by transcription factors MIBP1 and SEF-2 in the murine brain. *Mol. Cell. Biol.* 19:3736-3747.
- Ducy P., and Karsenty G. (1995). Two distinct osteoblast-specific cis-acting elements control expression of a mouse osteocalcin gene. *Mol. Cell. Biol.* 15:1858-1869.
- Ducy P., Starbuck M., Priemel M., Shen J., Pinero G., Geoffroy V., Amling M., and Karsenty G. (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. *Genes Dev.* 13:1025-1036.
- Ducy P. (2000). Cbfa1: a molecular switch in osteoblast biology. *Dev. Dyn.* 19:461-471.
- Karsenty G., and Wagner E.F. (2002). Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell.* 2:389-406.
- Meyers S., Lenny N., and Hiebert S.W. (1995). The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. *Mol. Cell. Biol.* 15:1974-1982.
- Müller J.M., Isele U., Metzger E., Rempel A., Moser M., Pscherer A., Breyer T., Holubarsch C., Buettner R., and Schüle R. (2000). FHL2, a novel tissue-specific coactivator of the androgen receptor. *EMBO J.* 19:359-369.
- Müller J.M., Metzger E., Greschik H., Bosserhoff A.K., Mercep L., Buettner R., and Schüle R. (2002). The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. *EMBO J.* 21:736-748.
- Parfitt A.M., Drezner M.K., Glorieux F.H., Kanis J.A., Malluche H., Meunier P.J., Ott S.M., and Recker R.R. (1987). Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J. Bone Miner. Res.* 2:595-610.

- Teitelbaum S.L. (2000). Bone resorption by osteoclasts. *Science*. 289:1504-1508.
- Yeung F., Law W.K., Yeh C.H., Westendorf J.J., Zhang Y., Wang R., Kao C., and Chung LW. (2002) Regulation of human osteocalcin promoter in hormone-independent human prostate cancer cells. *J. Biol. Chem.* 277:2468-2476.

Claims

1. A method for identifying a compound that promotes the activity of osteoblasts, comprising:

- (a) contacting at least one cell with a test compound *in vitro*;
- (b) determining an activity of the *Fhl2* gene or *Fhl2* protein in the at least one cell;
- (c) comparing the activity determined in (b) to the activity of the *Fhl2* gene or *Fhl2* protein in at least one control cell that has not been contacted with the test compound; and
- (d) selecting the test compound if the activity measured in (b) is significantly different from that in the at least one control cell.

2. A method according to claim 1, comprising:

- (a) contacting at least one cell with a test compound *in vitro*;
- (b) measuring the level of *Fhl2* expression in the at least one cell;
- (c) comparing the level of *Fhl2* expression measured in (b) to the level of *Fhl2* expression in at least one control cell that has not been contacted with the test compound; and
- (d) selecting the compound if the level of *Fhl2* expression measured in (b) is higher than that in the at least one control cell.

3. A method according to claim 1, comprising:

- (a) contacting at least one cell with a test compound *in vitro*;
- (b) measuring the amount of *Fhl2* protein in the nucleus of the at least one cell;
- (c) comparing the amount of *Fhl2* protein measured in (b) to the amount of *Fhl2* protein in the nucleus of the at least one control cell that has not been contacted with the test compound; and
- (d) selecting the compound if the level of *Fhl2* protein measured in (b) is higher than that in the control cell(s).

4. A method according to claim 1, comprising:

- (a) contacting a test compound with at least one cell *in vitro*;

- (b) determining the level of interaction between *Fhl2* protein and *Runx2* protein in the cell(s);
 - (c) comparing the level of interaction determined in (b) to the level of interaction between *Fhl2* protein and *Runx2* protein in at least one control cell that has not been contacted with the test compound; and
 - (d) selecting the compound if the level of interaction measured in (b) is significantly different from that in the control cell(s).
5. A method according to any one of claims 1 to 4 wherein the at least one cell is selected from the group consisting of primary osteoblasts, MC3T3-E1 cells, ROS17 cells and U2-OS cells.
6. A method for the preparation of a compound that is useful in the treatment of a bone disease, comprising:
- (a) identifying a compound by a method according to any one of claims 1 to 5; and
 - (b) synthesizing the compound.
7. A compound that is useful in the treatment of a bone disease wherein the compound is capable of promoting osteoblast activity by enhancing the expression of the *Fhl2* gene, promoting the translocation of *Fhl2* protein in the nucleus and/or modulating the interaction between *Fhl2* protein and *Runx2* protein.
8. A compound according to claim 7 wherein the compound is capable of enhancing signals mediated by Rho proteins.
9. The use of an *Fhl2* nucleic acid for the manufacture of a medicament for the treatment of a bone disease wherein the *Fhl2* nucleic acid is selected from the group consisting of
- (a) polynucleotides comprising the sequence as shown in SEQ ID NO:1;
 - (b) polynucleotides comprising a sequence which has an identity of at least 50% to the sequence as shown in SEQ ID NO:1;

- (c) polynucleotides hybridizing to the sequence as shown in SEQ ID NO:1 under stringent conditions;
 - (d) polynucleotides comprising a sequence which encodes a polypeptide having an amino acid sequence as shown in SEQ ID NO:2; and
 - (e) polynucleotides comprising a sequence which encodes a polypeptide having an amino acid sequence which has an identity of at least 70% to the amino acid sequence as shown in SEQ ID NO:2.
10. The use according to claim 9 wherein the *Fhl2* nucleic acid is a polynucleotide encoding a polypeptide having an amino acid sequence as shown in SEQ ID NO:2.
11. The use according to claim 9 or 10 wherein the *Fhl2* nucleic acid is a polynucleotide comprising the sequence as shown in SEQ ID NO:1.
12. The use according to any one of claims 9 to 11 wherein the bone disease is characterized by a decreased bone mass relative to that of non-diseased bone.
13. The use according to any one of claims 9 to 12 wherein the bone disease is osteoporosis.
14. The use according to any one of claims 9 to 13 wherein the *Fhl2* nucleic acid is overexpressed in osteoblasts.
15. A method of diagnosing a bone disease, comprising
- (a) determining *in vitro* the level of expression of the *Fhl2* gene in tissue from an individual; and
 - (b) comparing the level determined in (a) to the level of expression of the *Fhl2* gene in control tissue;
- so that if the level determined in (a) is lower than that of the control, the individual is diagnosed as exhibiting the bone disease.
16. A method according to claim 15 wherein the bone disease is osteoporosis.

17. The use of a transgenic non-human animal characterized by a decreased level of expression of the Fhl2 gene relative to that of the corresponding wild-type animal as an osteoporosis model.

18. The use according to claim 17 wherein the transgenic non-human animal is a knockout mouse.

19. A method for identifying a compound that promotes the activity of osteoblasts, comprising:

- (a) administering a test compound to a transgenic non-human animal characterized by a decreased level of expression of the Fhl2 gene relative to that of the corresponding wild-type animal;
- (b) determining an activity of the Fhl2 gene or Fhl2 protein;
- (c) comparing the activity determined in (b) to the activity of the Fhl2 gene or Fhl2 protein in a control animal that has not been contacted with the test compound; and
- (d) selecting the test compound if the activity measured in (b) is significantly different from that in the control animal.

Figure 1

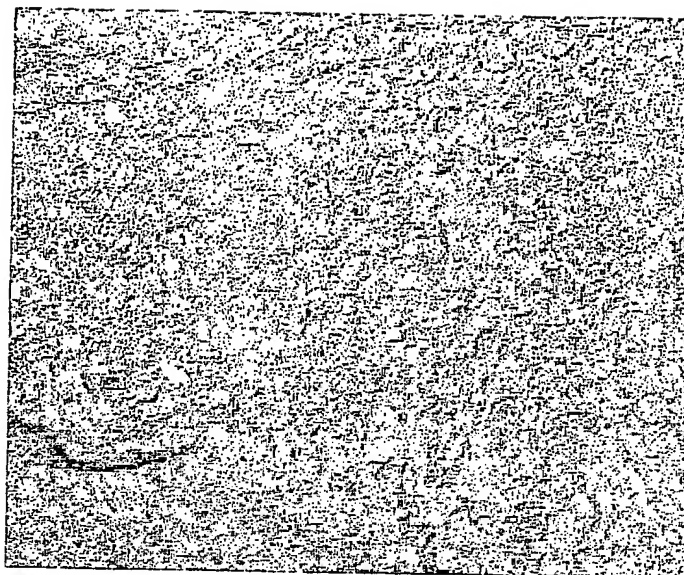
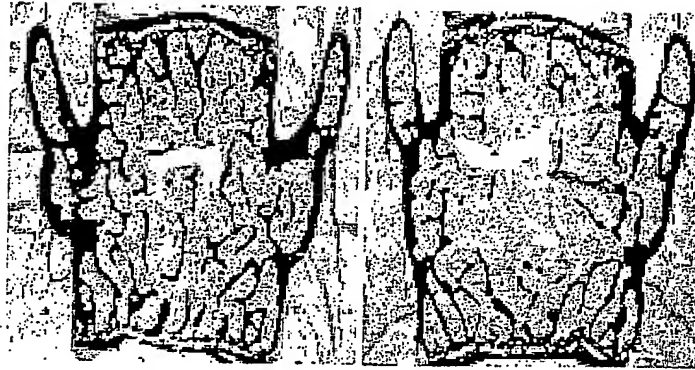
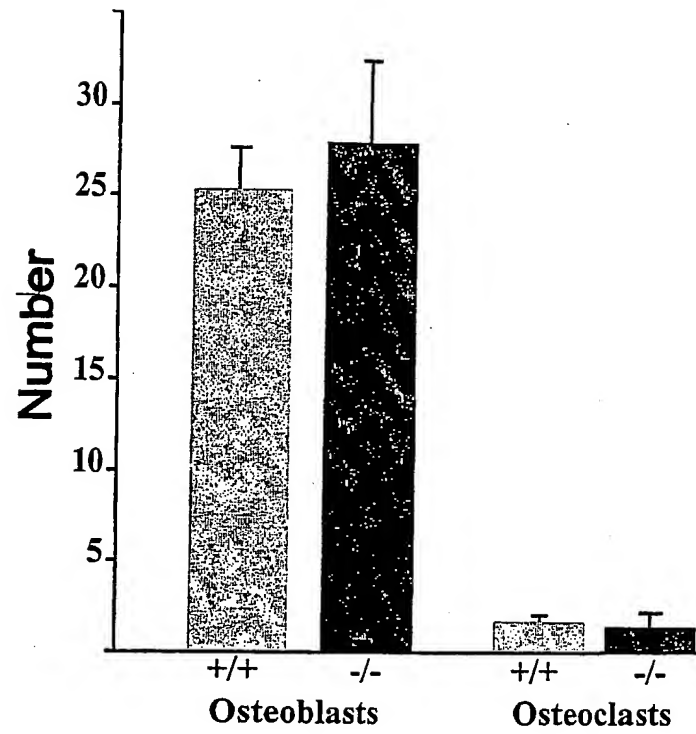


Figure 2



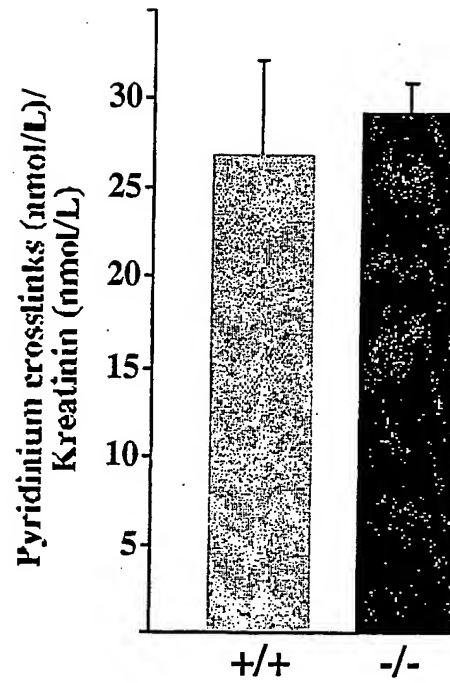
3/11

Figure 3



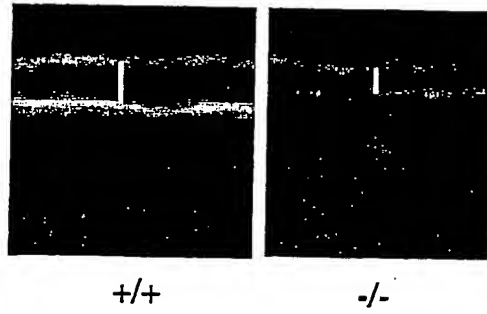
4/11

Figure 4



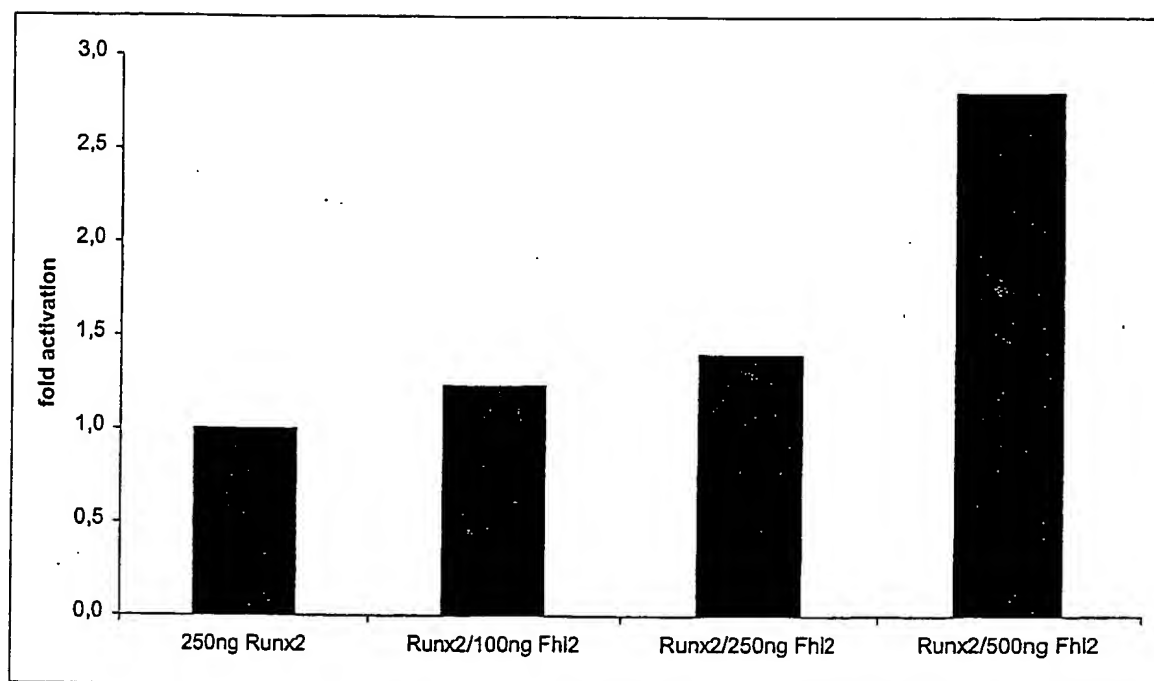
5/11

Figure 5



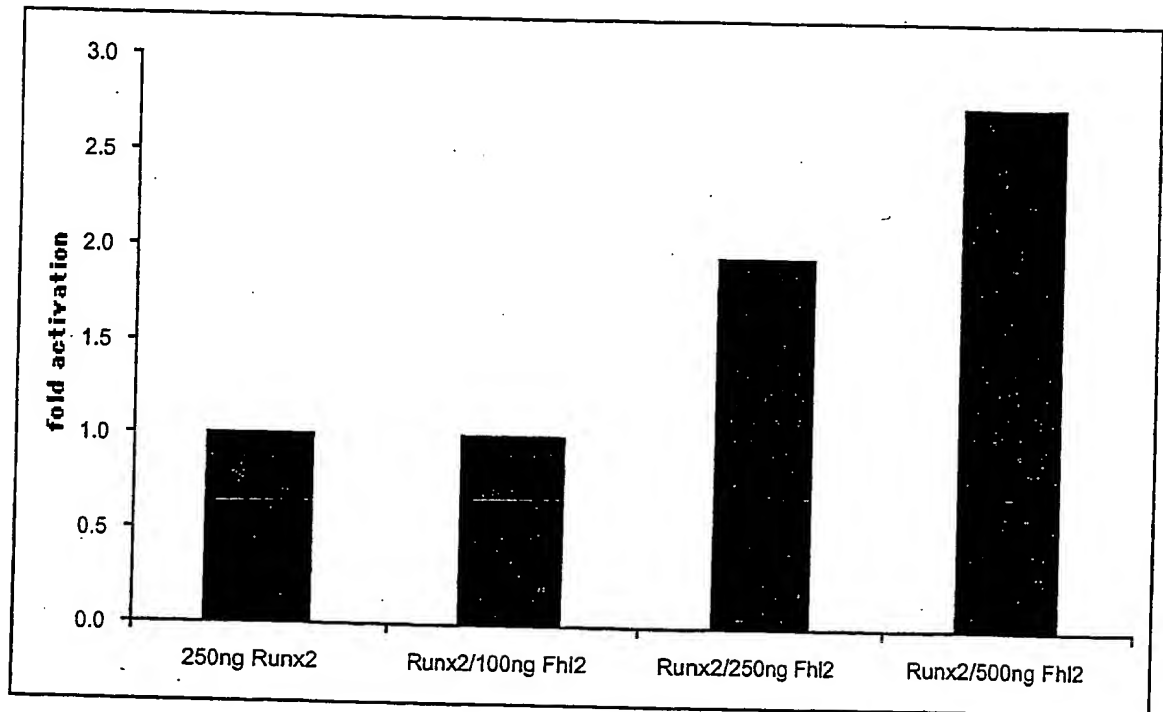
6/11

Figure 6



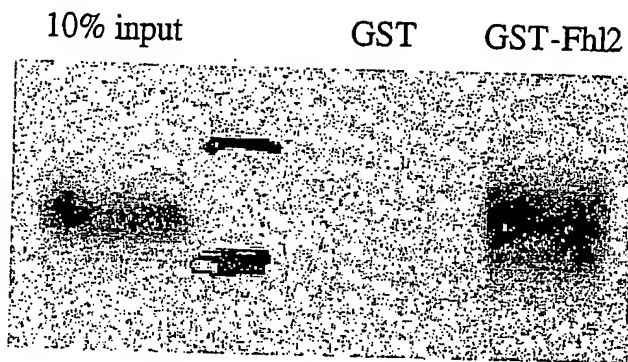
7/11

Figure 7



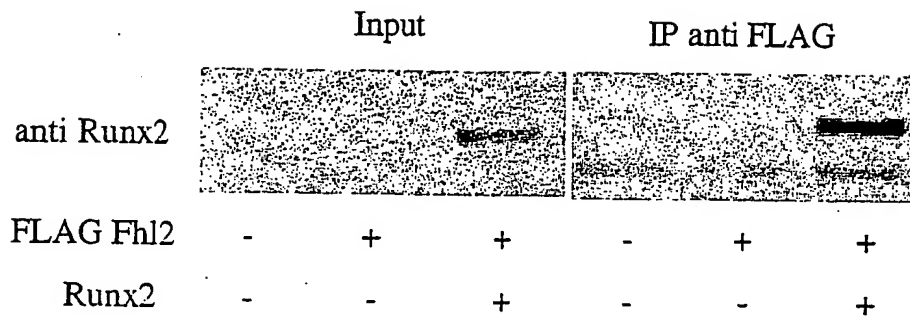
8/11

Figure 8



9/11

Figure 9



10/11

Figure 10

```
1  aggggtacggg  cggggaccgc  cgcagcccgg  ggcgggggca  cggcaaccgc  gaggcctggg
61  ggcgcccgcc  ccccgcgccc  cacgcccggg  gccagcgagc  cgaggcggtc  atctccttat
121 atgggtcaaat  gacacggcgg  ggtttctcga  gggcgggagc  tgcgcagcgc  tccactcggc
181 cggcagcggg  gccgcagcca  ccagccgccc  gcgcccctca  gcccgcgtcc  ggagtcctcg
241 gcccgctgcg  gtgccgtgag  tacctccaac  cccctgcgcc  ccggaggggg  gccgaggggc
301 ttagccacca  gggctcggaa  gtggggggcg  aatccgggtg  gagacccaag  gagagggggg
361 cagagccggg  gttggggaga  ctgtgggtga  aaactgtgtc  ttcctggaga  ctaggctggc
421 attttgactt  tgggacggag  tctcgctttg  tcgcccaggc  tggagtgcag  tggcacgata
481 tcagctcact  gcaagctcta  cctcttggtt  cacgccattc  tcctgcccc  gcctcccaag
541 tagctgggac  tacaggttgc  tgaaaagcca  ggagtcacaa  tgactgagcg  ctttgactgc
601 caccattgca  acgaatctct  ctttggaag  aagtacatcc  tgcgggagga  gagcccctac
661 tgcgtggtgt  gctttgagac  cctgttcgcc  aacacctgcg  aggagtgtgg  gaagcccatc
721 ggctgtgact  gcaaggactt  gtcttacaag  gaccggcact  ggcataaagc  ctgtttccac
781 tgctcgcagt  gcagaaactc  actggtggac  aagccctttg  ctgccaagga  ggaccagctg
841 ctctgtacag  actgctattc  caacgagtag  tcatccaagt  gccaggaatg  caagaagacc
901 atcatgccag  gtacccgcaa  gatggagtag  aagggcagca  gctggcatga  gacctgcttc
961 atctgccacc  gctgccagca  gccaatgga  accaagagtt  tcatcccaa  agacaatcag
1021 aatttctgtg  tgccctgcta  tgagaaacaa  catgccatgc  agtgcgttca  gtgcaaaaag
1081 cccatcacca  cgggaggggt  cacttacggg  gagcagccct  ggcacaagga  gtgcttcgtg
1141 tgcaccgcct  gcaggaagca  gctgtctggg  cagcgcttca  cagctcgcga  tgactttgcc
1201 tactgcctga  actgcttctg  tgacttgtat  gccagaaggt  gtgctgggtg  caccaacccc
1261 atcagcggac  ttggtggcac  aaaatacatc  tcctttgagg  aacggcagtg  gcataacgac
1321 tgctttaact  gtaagaagt  ctccctctca  ctggtggggc  gtggcttcct  cacagagagg
1381 gacgacatcc  tgtgccccga  ctgtgggaaa  gacatctgaa  ttcaacacag  agaagttgct
1441 gcttgtgatc  tcacacacag  atttttatgt  tttctttctc  acccaggcaa  tcttgcttct
1501 tggtttcttc  cagccacatt  gagactttct  tctagtgttt  ttcagtgata  ctcacgtttg
1561 cttaaaccct  ttagtgcttt  gtgatagttc  agtcccaggg  aaagagaaaa  ctgcctctag
1621 gccctaggtg  ggaagatggt  ttgaaatttt  tgtaatcgag  taaggcacac  ccaaatgtaa
1681 aaatcctttt  gaatgatgcc  ttataaaatc  tttctctcac  tgtctattta  agtgcaatta
1741 acatatgtca  cgaacttgaa  agttttctaa  actcaataag  gtaatgacca  gttgttattt
1801 acagctctgt  aacctccggt  tgcgtcaagt  ctaaaccaag  attatgtgac  ttgcaataaa
1861 gttattcaga  acagaaaaaa  aaaaaaaaaa  aa
```

Figure 11

A

```

atgactgagc gctttgactg ccaccattgc aacgaatctc tctttggcaa gaagtaçatc      60
ctgcggggagg agagccccta ctgcgtggtg tgctttgaga ccctgttcgc caacacctgc      120
gaggagtgtg ggaagcccat cggctgtgac tgcaaggact tgtcttacia ggaccggcac      180
tggcatgaag cctgtttcca ctgctcgag tgcagaaact cactggtgga caagcccttt      240
gctgccaagg aggaccagct gctctgtaca gactgctatt ccaacgagta ctcaccaag      300
tgccaggaat gcaagaagac catcatgcca ggtaccgcga agatggagta caagggcagc      360
agctggcatg agacctgctt catctgccac cgctgccagc agccaattgg aaccaagagt      420
ttcatcccca aagacaatca gaatttctgt gtgccttct atgagaaaca acatgccatg      480
cagtgcgttc agtgcaaaaa gcccatcacc acgggagggg tcaactaccg ggagcagccc      540
tggcacaagg agtgcttcgt gtgcaccgcc tgcaggaagc agctgtcttg gcagcgcttc      600
acagctcgcg atgactttgc ctactgcctg aactgcttct gtgacttgta tgccaagaag      660
tgtgctgggt gcaccaaccc catcagcgga cttggtggca caaaatacat ctcccttgag      720
gaacggcagt ggcataacga ctgctttaac tgtaagaagt gtcctctc actggtgggg      780
cgtggcttcc tcacagagag ggacgacatc ctgtgccccg actgtgggaa agacatctga      840

```

B

```

MTERFDCHHC NESLFGKKYI LREESPYCVV CFETLFANTC EECGKPIGCD CKDLSYKDRH
WHEACFHCSQ CRNSLVDPKF AAKEDQLLCT DCYSNEYSSK CQECKKTIMP GTRKMEYKGS
SWHETCFICH RCQOPIGTKS FIPKDNQNFV VPCYEKQHAM QCVQCKKPIT TGGVITYREQP
WHKECFVCTA CRKQLSGQRF TARDDFAYCL NCFCDLYAKK CAGCTNPISG LGGTKYISFE
ERQWHND CFN CKKCSLSLVG RGFLTERDDI LCPDCGKDI

```

10/561902

SEQUENCE LISTING

IAP9 Rec'd PCT/PTO 21 DEC 2005

<110> Universitätsklinikum Freiburg

<120> Modulation of Osteoblast Activity by Fh12

<130> Fh12

<160> 3

<170> PatentIn version 3.1

<210> 1

<211> 840

<212> DNA

<213> homo sapiens

```

<400> 1
atgactgagc gctttgactg ccaccattgc aacgaatctc tctttggcaa gaagtacatc      60
ctgcgggagg agagccccta ctgcgtggtg tgctttgaga ccctgttcgc caacacctgc      120
gaggagtgtg ggaagcccat cggctgtgac tgcaaggact tgtcttaca ggaccggcac      180
tgcatgaag cctgtttcca ctgctcgag tgcagaaact cactggtgga caagcccttt      240
gctgccaagg aggaccagct gctctgtaca gactgctatt ccaacgagta ctcattcaag      300
tgccaggaat gcaagaagac catcatgcca ggtacccgca agatggagta caagggcagc      360
agctggcatg agacctgctt catctgccac cgctgccagc agccaattgg aaccaagagt      420
ttcatcccca aagacaatca gaatttctgt gtgccctgct atgagaaaca acatgccatg      480
cagtgcgttc agtgcaaaaa gcccatcacc acgggagggg tcaattaccg ggagcagccc      540
tggcacaagg agtgcttcgt gtgcaccgcc tgcaggaagc agctgtcttg gcagcgcttc      600
acagctcgcg atgactttgc ctactgcctg aactgcttct gtgacttgta tgccaagaag      660
tgtgctgggt gcaccaaccc catcagcgga cttggtggca caaaatacat ctcctttgag      720
gaacggcagt ggcataacga ctgctttaac tgtaagaagt gctccctctc actggtgggg      780
cgtggcttcc tcacagagag ggacgacatc ctgtgccccg actgtgggaa agacatctga      840

```

<210> 2

<211> 279

<212> PRT

<213> homo sapiens

<400> 2

Met Thr Glu Arg Phe Asp Cys His His Cys Asn Glu Ser Leu Phe Gly
1 5 10 15

Lys Lys Tyr Ile Leu Arg Glu Glu Ser Pro Tyr Cys Val Val Cys Phe
20 25 30

Glu Thr Leu Phe Ala Asn Thr Cys Glu Glu Cys Gly Lys Pro Ile Gly
35 40 45

Cys Asp Cys Lys Asp Leu Ser Tyr Lys Asp Arg His Trp His Glu Ala
50 55 60

Cys Phe His Cys Ser Gln Cys Arg Asn Ser Leu Val Asp Lys Pro Phe
65 70 75 80

Ala Ala Lys Glu Asp Gln Leu Leu Cys Thr Asp Cys Tyr Ser Asn Glu
85 90 95

Tyr Ser Ser Lys Cys Gln Glu Cys Lys Lys Thr Ile Met Pro Gly Thr
100 105 110

Arg Lys Met Glu Tyr Lys Gly Ser Ser Trp His Glu Thr Cys Phe Ile
115 120 125

Cys His Arg Cys Gln Gln Pro Ile Gly Thr Lys Ser Phe Ile Pro Lys
130 135 140

Asp Asn Gln Asn Phe Cys Val Pro Cys Tyr Glu Lys Gln His Ala Met
145 150 155 160

Gln Cys Val Gln Cys Lys Lys Pro Ile Thr Thr Gly Gly Val Thr Tyr
165 170 175

Arg Glu Gln Pro Trp His Lys Glu Cys Phe Val Cys Thr Ala Cys Arg
180 185 190

Lys Gln Leu Ser Gly Gln Arg Phe Thr Ala Arg Asp Asp Phe Ala Tyr
195 200 205

Cys Leu Asn Cys Phe Cys Asp Leu Tyr Ala Lys Lys Cys Ala Gly Cys
210 215 220

Thr Asn Pro Ile Ser Gly Leu Gly Gly Thr Lys Tyr Ile Ser Phe Glu
225 230 235 240

Glu Arg Gln Trp His Asn Asp Cys Phe Asn Cys Lys Lys Cys Ser Leu
245 250 255

Ser Leu Val Gly Arg Gly Phe Leu Thr Glu Arg Asp Asp Ile Leu Cys
 260 265 270

Pro Asp Cys Gly Lys Asp Ile
 275

<210> 3

<211> 1892

<212> DNA

<213> homo sapiens

<400> 3

```

agggtacggg ccgggaccgc cgcagcccgg ggcgggggca cggcaaccgc gaggcctggg      60
ggcggccgcc ccccgcgccc caccgccggg gccagcgagc cgaggcgtgc atctccttat      120
atggtcaaat gacacggcgg ggtttctcga ggcggggagc tgcgcagcgc tccactcggc      180
cggcagcggg gccgcagcca ccagccgccc gcgccctcca gcccgtccg ggagtccccg      240
gcccgtgcg gtgccgtgag tacctccaac cccctgcgcc ccggaggag gagagggggc      300
ttagccacca gggctcggaa gtggggggccg aatccgggtc gagaccaag gagagggggg      360
cagagccgga gttggggaga ctgtggctga aaactgtgtc ttcctggaga ctaggtgggc      420
atcttgactt tgggacggag tctcgcttgg tcgcccaggc tggagtgcag tggcacgac      480
tcagctcact gcaagctcta cctcttggtt caccgcatc tcctgcccc gctcccaag      540
tagctgggac tacaggttgc tgaaaagcca ggagtcaaaa tgactgagcg ctttgactgc      600
caccattgca acgaatctct ctttggaag aagtacatcc tgcgggagga gagcccctac      660
tgcgtgggtg gctttgagac cctgttcgcc aacacctgcg aggagtgtgg gaagcccatc      720
ggctgtgact gcaaggactt gtcttacaag gaccggcact ggcataaagc ctgtttccac      780
tgctcgcagt gcagaaactc actggtggac aagcccttgg ctgccaagga ggaccagctg      840
ctctgtacag actgctattc caacgagtac tcatccaagt gccaggaatg caagaagacc      900
atcatgccag gtaccgcaa gatggagtac aagggcagca gctggcatga gacctgcttc      960
atctgccacc gctgccagca gccaatgga accaagagtt tcatcccaa agacaatcag      1020
aatttctgtg tgccctgcta tgagaaacaa catgccatgc agtgcgttca gtgcaaaaag      1080
cccatcacca cgggaggggt cacttaccgg gagcagccct ggcacaagga gtgcttcgtg      1140
tgcaccgcct gcaggaagca gctgtctggg cagcgcttca cagctcgcga tgactttgcc      1200
tactgcctga actgcttctg tgacttgtat gccaaagagt gtgctgggtg caccaacccc      1260
atcagcggac ttggtggcac aaaatacatc tcctttgagg aacggcagtg gcataacgac      1320
tgctttaact gtaagaagtg ctccctctca ctggtggggc gtggcttcct cacagagagg      1380
gacgacatcc tgtgccccga ctgtgggaaa gacatctgaa ttcaacacag agaagttgct      1440

```

gcttgatc tcacacacag atttttatgt tttctttctc acccaggcaa tcttgcttc	1500
tggtttcttc cagccacatt gagactttct tctagtgtt ttcagtgata ctcacgttg	1560
cttaaaccct ttagtgcttt gtgatagttc agtcccaggg aaagagaaaa ctcgacctag	1620
gccctaggtg ggaagatggt ttgaaatfff tgtaatcgag taaggcacac ccaaattgaa	1680
aaatcctfff gaatgatgcc ttataaatc tttctctcac tgtctattta agtgcaatta	1740
acatatgtca cgaacttgaa agttttctaa actcaataag gtaatgacca gttgttattt	1800
acagctctgt aacctcccg tgcgtcaagt ctaaaccaag attatgtgac ttgcaataaa	1860
gttattcaga acagaaaaaa aaaaaaaaaa aa	1892

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/006798

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, MEDLINE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MÜLLER JUDITH M ET AL: "The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus." THE EMBO JOURNAL. ENGLAND 15 FEB 2002, vol. 21, no. 4, 15 February 2002 (2002-02-15), pages 736-748, XP001156130 ISSN: 0261-4189 cited in the application	7,8
Y	abstract page 737, column 2, paragraph 2 page 739, column 2, paragraph 2; figure 7 page 745, column 1, last paragraph - column 2, last paragraph; figure 3A page 746, column 1, paragraph 3 page 746, column 2, paragraph 2 ----- -/-	1-6

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the International filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the International filing date but later than the priority date claimed

T later document published after the International filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the International search

22 September 2004

Date of mailing of the International search report

30/09/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Bigot-Maucher, C

INTERNATIONAL SEARCH REPORT

 International Application No
 PCT/EP2004/006798

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>AMAAR YOUSEF G ET AL: "Insulin-like growth factor-binding protein 5 (IGFBP-5) interacts with a four and a half LIM protein 2 (FHL2)"</p> <p>JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 14, 5 April 2002 (2002-04-05), pages 12053-12060, XP001153236</p> <p>ISSN: 0021-9258</p>	7,8
Y	<p>abstract</p> <p>page 12054, column 1, paragraph 2</p> <p>page 12058, column 1, paragraph 1</p> <p>page 12059, column 1, paragraph 4</p>	1-6,19
Y	<p>JOHANNESSEN MONA ET AL: "Activation of the coactivator four-and-a-half-LIM-only protein FHL2 and the c-fos promoter through inhibition of protein phosphatase 2A."</p> <p>BIOCHEMICAL PHARMACOLOGY, vol. 65, no. 8, 15 April 2003 (2003-04-15), pages 1317-1328, XP001153237</p> <p>ISSN: 0006-2952</p> <p>abstract</p>	1-6
Y	<p>CHAN K K ET AL: "Molecular cloning and characterization of FHL2, a novel LIM domain protein preferentially expressed in human heart"</p> <p>GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, ELSEVIER SCIENCE PUBLISHERS, BARKING, GB, vol. 210, no. 2, 14 April 1998 (1998-04-14), pages 345-350, XP004118187</p> <p>ISSN: 0378-1119</p> <p>figure 1</p>	9
X	<p>KONG Y ET AL: "Cardiac-specific LIM protein FHL2 modifies the hypertrophic response to beta-adrenergic stimulation."</p> <p>CIRCULATION. UNITED STATES 5 JUN 2001, vol. 103, no. 22, 5 June 2001 (2001-06-05), pages 2731-2738, XP001153299</p> <p>ISSN: 1524-4539</p> <p>cited in the application</p>	7,8
Y	<p>page 2732, columns 1-2, paragraph BRIDGING</p>	19
Y	<p>WO 99/51727 A (SCHMITT ARMIN ;SPECHT THOMAS (DE); DAHL EDGAR (DE); HINZMANN BERND) 14 October 1999 (1999-10-14) page 163, paragraph 9 - page 164, paragraph 4</p>	9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/006798

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LAI C F ET AL: "Four and a half Lim protein 2 (FHL2) stimulates osteoblast proliferation and differentiation." JOURNAL OF BONE AND MINERAL RESEARCH, vol. 17, no. Suppl 1, September 2002 (2002-09), page S129, XP009020682 Twenty-Fourth Annual Meeting of the American Society for Bone and Mineral Research; San Antonio, Texas, USA; September 20-24, 2002 ISSN: 0884-0431 (ISSN print) abstract -----	1-6
A	US 6 333 312 B1 (KUBERASAMPATH THANGAVEL ET AL) 25 December 2001 (2001-12-25) column 2, paragraph 5; examples 2-7 column 3, paragraphs 1,3,4 -----	1-19
A	VAUGHAN TANYA ET AL: "Alleles of RUNX2/CBFA1 gene are associated with differences in bone mineral density and risk of fracture." JOURNAL OF BONE AND MINERAL RESEARCH, vol. 17, no. 8, August 2002 (2002-08), pages 1527-1534, XP009021473 ISSN: 0884-0431 (ISSN print) abstract -----	1-19

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)